Impact of effector cell differentiation on CD4+ T cells that evade negative selection by a self-peptide

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Abstract
We have used a transgenic mouse system to examine how differing reactivities of TCRs expressed by naive versus effector cells can shape the functional potential of autoreactive CD4+ T cells. Transgenic mice expressing TCRs that exhibit either high (TS1) or low (TS1(SW)) reactivity toward the I-Eα-restricted determinant S1 from the influenza virus PR8 hemagglutinin (HA) were mated with transgenic mice expressing HA under the control of different promoters. HACII mice express HA driven by an MHC class II promoter, and both the TS1 and TS1(SW) TCRs underwent substantial deletion in this background. HA104 mice express HA driven by an SV40 promoter, and the highly reactive TS1 TCR was substantially deleted. By contrast, the less reactive TS1(SW) TCR underwent little or no deletion in TS1(SW) × HA104 mice, although CD5 up-regulation indicated that they had interacted with the S1 self-peptide. In adoptive transfer studies, naive CD4+ T cells expressing the TS1(SW) TCR failed to proliferate in response to the S1 peptide in HA104 mice, and were inefficient at providing help for HA-specific antibody responses. However, effector CD4+ T cells generated from TS1(SW) × HA104 mice acquired the ability to proliferate in response to the S1 peptide in HA104 mice, and were as efficient as CD4+ T cells expressing the high reactivity TS1 TCR in helping HA-specific antibody responses. Collectively, these studies demonstrate a basis by which CD4+ T cells expressing TCRs with low reactivity toward self-peptides can evade negative selection and acquire enhanced autoreactivity following activation by a cross-reactive antigen.

Introduction
CD4+ T cells recognize MHC class II molecules presenting peptides from foreign or self-antigens, and the specificity of this interaction is dictated by the α/β heterodimer of the TCR (1). The ability of an individual MHC molecule to present diverse peptides, and the use of gene rearrangement to generate TCRs, each ensures that the immune system has the capacity to recognize an unknown universe of foreign antigens. However, self-antigens are also highly diverse and can be expressed in varying amounts and by different cell types, and TCRs can possess differing reactivities even for the same peptide–MHC complex. How the diversity with which CD4+ T cells can recognize self-peptides affects the regulation of autoreactive CD4+ T cells is not yet fully understood.

There is good evidence that the reactivity that a TCR possesses for self-peptide–MHC complexes is an important parameter in directing T cell development (2). Weak interactions with peptide–MHC complexes are required to promote positive selection of thymocytes in fetal thymic organ culture (3). In addition, thymocytes undergoing positive selection in vivo also exhibit phenotypic changes (such as up-regulation of CD69 and CD5) that reflect non-deletional interactions with self-peptide–MHC complexes (4, 5). By contrast, high concentrations of agonist peptides can induce deletion of thymocytes in vitro (6–8), and agonist self-peptides have been shown to induce thymocytes to undergo tolerance induction in vivo by inducing deletion or CD25+ regulatory T cell formation (9–11). Still, there likely exists a range of reactivities that TCRs can exhibit toward self-peptide–MHC complexes that lies between the thresholds required to induce positive selection versus tolerance induction, and whether these cells remain in a quiescent state through ‘ignorance’ or by more active processes such as anergy induction is still being...
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determined. CD4+ T cells that evade tolerance induction despite reactivity toward self-peptide–MHC complexes have the potential for autoreactivity if they become activated, for example by a microbial antigen bearing a cross-reactive epitope (12). In this regard, whether the increased sensitivity of effector T cells to activation through the TCR can cause autoreactive T cells that evade deletion to acquire the ability to proliferate and/or mediate effector function in response to the self-peptide–MHC complexes remains to be determined (13–16).

We have used transgenic mice expressing the influenza virus hemagglutinin (HA) under the control of different promoters, and co-expressing MHC class II-restricted TCRs with high or low reactivity for the HA-derived peptide S1, to examine how variations in antigen expression and TCR reactivity affect the formation and functional potential of autoreactive CD4+ T cells. In one setting, CD4+ T cells were found to evade deletion by S1 peptide, while also exhibiting evidence (CD6 up-regulation) that they had interacted with S1 during their development. We show that these CD4+ T cells that evade negative selection because of their low reactivity for S1 can be activated by a cross-reactive viral antigen, and that effector cells acquire the capacity to proliferate in response to the S1 self-peptide and to provide efficient B cell help. These studies show that the development of an effector phenotype may allow CD4+ T cells that evaded tolerance induction due to low reactivity toward peptide–MHC complexes to acquire overt autoreactivity and participate in anti-self-immune responses.

Methods

Mice

HA104, HACII, TS1 and TS1(SW) mice were described previously (9, 11, 17). All mouse lineages were backcrossed to BALB/c mice at least 10 generations and maintained in sterile microisolators at the Wistar Institute Animal Facility. All studies have been reviewed and approved by the Wistar Institute Animal Care and Use Committee.

Flow cytometry

Thymocytes and lymph node (LN) cells were analyzed on FACSCalibur or FACSCan flow cytometers (Becton Dickinson, San Jose, CA, USA). Data analysis was carried out with the computer program CellQuest (Becton Dickinson). The following antibodies were used for analysis: PE–anti-CD4 (L3T4/ RM4-5), PE–anti-CD5 (Ly-1/53-7.3), PE–anti-CD86 (B7.2/GL1), biotin–anti-I-A<sup>d</sup> (AMS-32.1), biotin–anti-V<sub>A</sub>3 (KT50), all from BD PharMingen (San Diego, CA, USA), and biotin–6.5 (18). Streptavidin-Red670 (Gibco-BRL, Gaithersburg, MD, USA) was used to detect biotinylated reagents. In experiments requiring purified subpopulations (6.5+ CD4+ or V<sub>A</sub>8.3+ V<sub>B</sub>10+ CD4+), cells were sorted using an EPICS Elite flow cytometer (Coulter Corporation, Miami, FL, USA).

Carboxyfluorescein succinimidyl labeling

The carboxyfluorescein succinimidyl (CFSE)-labeling protocol was previously described (19). Briefly, LN cell suspension was washed into serum-free Iscove's modified Dulbecco's medium (IMDM) incubated with 5 mM CFSE (Molecular Probes, Eugene, OR, USA) at 1 x 10<sup>6</sup> cells ml<sup>-1</sup> for 5 min and then incubated with 50% FCS and washed into serum-free IMDM.

Adoptive transfers

A total of 5–10 x 10<sup>6</sup> or 3–5 x 10<sup>6</sup> CFSE-labeled LN cells from TS1, TS1(SW) or TS1(SW) x HA104 mice were injected into the tail vein of unmanipulated or sublethally irradiated (700 rad) recipients. Three or seven days later axillary, brachial and inguinal LNs of the recipient mice were obtained and cell suspensions stained for flow cytometry.

Complement-mediated depletion of CD8+ T cells

LN single-cell suspensions were incubated with culture supernatant of the anti-CD8 hybridoma 3.168.8 at 1 x 10<sup>7</sup> cells ml<sup>-1</sup> for 30 min on ice. The unbound antibodies were removed by washing the cells in serum-free IMDM. Low-Tox-M rabbit complement (Cedarlane, Hornby, Ontario, Canada) was added at the concentration and for the duration indicated by the manufacturer for lysis of mouse lymphocytes. Efficiency of CD8+ T cell depletion was typically 98% when assessed by flow cytometry.

Generation of effector cells and Th<sub>1</sub> differentiation

CD8-depleted LN cells from TS1(SW) or TS1(SW) x HA104 mice were re-suspended in IMDM supplemented with 10% FCS at a concentration of 1 x 10<sup>6</sup> cells ml<sup>-1</sup> and stimulated with 1 µM S1(SW) peptide (SFEKFEIFPKT; synthesized and HPLC purified by the Wistar Institute Peptide Synthesis Facility) in the presence of syngeneic antigen-presenting cells (APCs) (5 x 10<sup>5</sup> cells ml<sup>-1</sup>) and IL-2 (10 ng ml<sup>-1</sup>). Cultures were expanded with fresh medium containing IL-2 after 3 days. At day 6 of culture, cells were washed and allowed to rest for an additional 3 days in IMDM supplemented with 10% FCS. For Th<sub>1</sub> differentiation, additional cytokines and/or neutralizing antibodies were added in the initial culture: IL-4 (10 U ml<sup>-1</sup> final concentration) from the cell line X-4 and anti-IFN-γ [XMG1.2, American Type Tissue Collection (ATCC), Manassas, VA, USA; final concentration 10 µg ml<sup>-1</sup>] were added for Th<sub>2</sub> differentiation, whereas anti-IL-4 (11B11, ATCC; final concentration 10 µg ml<sup>-1</sup>) and rIL-12 (a kind gift from Dr. G. Trinchieri; 0.5 ng ml<sup>-1</sup> final concentration) were added for Th<sub>1</sub> differentiation. At day 9 of culture, rested cells were harvested and purified over Lympholyte M (Cedarlane) before use.

Proliferation assays

Responder cells (5 x 10<sup>4</sup> cells per well) were cultured with 5 x 10<sup>5</sup> cells per well irradiated syngeneic BALB/c splenocytes and graded doses of S1 (SFERFEIFPKT and S1(SW) (SFEKFEIFPKT) peptides (synthesized and HPLC purified by the Wistar Institute Peptide Synthesis Facility) in 200-µl volumes of IMDM supplemented with 10% FBS in 96-well flat-bottom plates. Seventy-two hours after activation, cultures were pulsed with 0.5 µCi per well of [3H]-thymidine and harvested 16 h later. In some experiments, CFSE-labeled CD8-depleted LN cells were used as responder cells and division was assessed by flow cytometry.
**Viruses and immunizations**

Influenza virus PR8 [A/Puerto Rico/8/34 (H1N1)] and J1 (a reassortant of PR8 containing the serologically non-cross-reactive H3 subtype HA) were grown in the allantoic cavity of 10-day-old fertilized chicken eggs, purified by sucrose gradient centrifugation and titered by chicken red blood cell agglutination. To generate virus-primed B cells, BALB/c mice were immunized intraperitoneally with 1000 hemagglutinating units (HAU) of PR8 virus and used in experiments 8 weeks later.

**B cell purification**

Splenocytes from PR8-primed BALB/c mice were incubated with biotinylated anti-CD4 and anti-CD8 antibodies for 30 min on ice. Streptavidin MACS MicroBeads (MiltenyiBiotech, Auburn, CA, USA) were used for magnetic labeling of cells bound to the biotinylated antibodies. MACS separation columns were then used to purify B cells, using the protocol recommended by the manufacturer (MiltenyiBiotech).

HA-specific antibody ELISA

ELISAs using PR8 and J1 viruses were previously described (20). Briefly, polyvinyl 96-well round-bottom plates (Costar, Cambridge, MA, USA) were coated overnight with virus at a concentration of 1000 HAU ml\(^{-1}\) in PBS plus 0.08% NaN\(_3\), and incubated at 4°C overnight and then blocked with 1% BSA in PBS plus 0.08% NaN\(_3\). Mouse serum was added following serial dilution and incubated for 90 min at room temperature. Bound serum antibody was detected using AP-conjugated goat anti-mouse IgG antibody (1 µg ml\(^{-1}\) in 1% BSA in PBS plus 0.08% NaN\(_3\). Southern Biotechnology Associates, Birmingham, AL, USA). Plates were developed using p-nitrophenyl phosphate (Pierce, Rockford, IL, USA) and absorbance was read at 405/650 nm using a microplate reader. HA specificity of the antibody was determined by the difference between absorbance of the PR8- versus J1-coated wells.

**Results**

**The TS1 and TS1(SW) TCRs exhibit reciprocal reactivities toward analogs of the S1 determinant**

The major I-E\(^d\)-restricted T cell determinant from the PR8 HA that is recognized in BALB/c mice is termed S1, and can be represented by amino acids 110–120 of the HA polypeptide (SFERFEIFPKE) (21). The influenza virus strain A/SW/33 (SW) contains an analog of the S1 determinant, termed S1(SW), that differs from S1 by two substitutions (R to K at residue 113 and E to T at residue 120). Two lineages of TCR transgenic mice have previously been created from mice immunized with PR8 or SW viruses: one of these (termed TS1 mice) was generated using TCR sequences from an S1-specific CD4+ T cell clone (18). In addition, the clonotypic TCR expressed by TS1 mice can be recognized by the monoclonal antibody 6.5. The second lineage of TCR transgenic mice [termed TS1(SW) mice] was generated using TCR sequences from an S1(SW)-specific CD4+ T cell hybridoma (11, 22). The TS1(SW) TCR uses V\(\alpha\)8.3/V\(\beta\)10 variable regions, allowing expression of the clonotypic TCR to be assessed based on V\(\alpha\)8.3 expression.

To determine the relative reactivities of each of these TCRs for the S1 and S1(SW) determinants, we used cell sorting to purify 6.5+ CD4+ T cells from TS1 mice and V\(\alpha\)8.3+ CD4+ T cells from TS1(SW) mice, which were then stimulated with graded doses of either S1 or S1(SW) peptides and proliferation was assessed. CD4+ T cells expressing the TS1 and TS1(SW) TCRs exhibited comparable reactivities toward their cognate S1 or S1(SW) peptides, with half-maximal stimulation values in the sub-micromolar range that is typical of stimulation by agonist peptides (23). They were also in each case weakly cross-reactive with the respective analog peptide; the 6.5+ CD4+ T cells from TS1 mice required ~30-fold higher concentrations of the S1(SW) peptide than S1 to achieve comparable proliferation, and the V\(\alpha\)8.3+ CD4+ T cells from TS1(SW) mice required ~100-fold higher concentrations of S1 than S1(SW) peptide (Fig. 1A).

We also examined how the differing reactivities of the TS1 and TS1(SW) TCRs can affect the ability of CD4+ T cells to proliferate in response to S1 peptide in vivo. CFSE-labeled LN cells from TS1 and TS1(SW) mice were transferred into HA transgenic mice expressing the PR8 HA (including S1 peptide) as a neo-self-antigen. We used two different lineages of HA transgenic mice for these studies: In HAC11 mice, a MHC class II promoter targets PR8 HA expression directly to APCs, and PR8 HA can be detected on the surface of B cells and dendritic cells (17, 24). In HA104 mice, HA expression is driven by the SV40 early-region promoter/enhancer and HA mRNA is widely expressed, although PR8 HA cannot be detected as a cell-surface antigen on APCs (24, 25). The 6.5+ CD4+ T cells from TS1 mice remained undivided following adoptive transfer into BALB/c mice, but underwent extensive division in response to the S1 peptide in both HAC11 and HA104 mice. Moreover, they divided more extensively in HAC11 mice than in HA104 mice, based on their lower CFSE levels (Fig. 1B). The V\(\alpha\)8.3+ CD4+ T cells from TS1(SW) mice also failed to proliferate following transfer into BALB/c mice, but proliferated in response to S1 peptide in HAC11 mice, although to a lesser extent than was observed for 6.5+ CD4+ T cells from TS1 mice (Fig. 1B). Notably, the V\(\alpha\)8.3+ CD4+ T cells from TS1(SW) mice remained undivided in HA104 mice, whereas the 6.5+ CD4+ T cells from TS1 mice had undergone extensive division in this setting.

Altogether, these data show that differences in the presentation of the S1 peptide in HAC11 versus HA104 mice affect the ability of the S1 neo-self-peptide to induce proliferation of CD4+ T cells expressing the TS1 and TS1(SW) TCRs, and that targeting expression of the S1 peptide to APCs in HAC11 mice enhances the ability of S1 peptide to induce proliferation of CD4+ T cells expressing the 6.5 TCR. In addition, the lower intrinsic reactivity of the TS1(SW) TCR for the S1 peptide limits its ability to proliferate in response to the S1 peptide in vivo, to the extent that it induces little or no proliferation of CD4+ T cells in response to the S1 peptide in HA104 mice.

**CD4+ TS1(SW) T cells evade deletion due to the low reactivity of TS1(SW) TCR for S1 peptide expressed in HA104 mice**

We have previously reported that CD4SP thymocytes expressing the TS1 TCR are subjected to extensive deletion
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Fig. 1. The reactivity of TS1 and TS1(SW) TCRs toward the S1 and S1(SW) determinants. (A) FACS-purified 6.5+ CD4+ LN cells from TS1 mice (open symbols) and V8.3+ CD4+ LN cells from TS1(SW) mice (filled symbols) were stimulated with graded doses of S1 (squares) or S1(SW) (circles) peptides. Proliferation was determined by [3H]-thymidine incorporation. Averages ± SD are of triplicate wells from a single experiment. Data are representative of three independent experiments. (B) A total of 1 × 10^6 CFSE-labeled LN cells from TS1 mice or TS1(SW) mice were adoptively transferred into BALB/c, HA104 and HACII hosts. After the indicated amount of time, lymphocytes from each host were stained with either anti-CD4 and 6.5 antibody (TS1 cells) or anti-CD4 and anti-V8.3 antibody (TS1(SW) cells). Histograms indicate CFSE levels of 6.5+ CD4+ cells (CD4+ TS1) or V8.3+ CD4+ [CD4+ TS1(SW)] in BALB/c, HA104 and HACII hosts at the indicated number of days post-transfer and are representative of three independent experiments.

when they develop in the presence of the S1 peptide in TS1 × HA104 and TS1 × HACII mice (9, 11). We wanted here to determine whether thymocytes expressing the TS1(SW) TCR might evade deletion in either TS1(SW) × HA104 or TS1(SW) × HACII mice due to its lower intrinsic reactivity for S1 peptide. The thymi of TS1(SW) × HACII mice had substantially reduced cellularity and reduced numbers of CD4SP thymocytes relative to mice that do not contain the S1 peptide. Moreover, V8.3+ CD4SP thymocytes were virtually undetectable in TS1(SW) × HACII mice (Fig. 2A). Thus, interactions with the S1 peptide in TS1(SW) × HACII mice induce efficient deletion of clonotypic V8.3+ CD4+ T cells despite the lower intrinsic reactivity of the TS1(SW) TCR for the S1 peptide.

By contrast, the thymi of TS1(SW) × HA104 and TS1(SW) mice were similar in terms of total cellularity and CD4SP thymocyte number [Fig. 2A and B; an apparent reduction in the numbers of CD4SP thymocytes expressing the clonotypic TCR between TS1(SW) × HA104 and TS1(SW) mice did not reach statistical significance]. Likewise, TS1(SW) × HA104 and TS1(SW) mice contained similar numbers of V8.3+ CD4+ T cells (data not shown). In the case of the TS1(SW) TCR, then, its low reactivity for the S1 peptide prevents it from being subjected to deletion by the S1 peptide as it is presented in HA104 mice, unlike the substantial deletion of the higher reactivity TS1 TCR.

We were interested to determine whether CD4SP thymocytes and CD4+ T cells expressing the clonotypic TCR in TS1(SW) × HA104 mice exhibited phenotypic changes that might indicate that they had interacted with the S1 peptide, even though they undergo little or no deletion. No differences in the expression of CD44, CD69 or CD45RB were found between TS1(SW) × HA104 and TS1(SW) × HACII mice (data not shown). As previously reported, no differences were found in CD25 expression in V8.3+ CD4+ T cells from TS1(SW) mice (Fig. 2C). Although the vast majority was subjected to deletion, the few V8.3+ CD4+ T cells from TS1(SW) mice that reached statistical significance [Fig. 2B; a total of 1 × 10^6 CFSE-labeled LN cells from TS1(SW) mice were adoptively transferred into BALB/c, HA104 and HACII hosts. After the indicated amount of time, lymphocytes from each host were stained with either anti-CD4 and 6.5 antibody (TS1 cells) or anti-CD4 and anti-V8.3 antibody (TS1(SW) cells). Histograms indicate CFSE levels of 6.5+ CD4+ cells (CD4+ TS1) or V8.3+ CD4+ [CD4+ TS1(SW)] in BALB/c, HA104 and HACII hosts at the indicated number of days post-transfer and are representative of three independent experiments.].

CD5 has previously been shown to be up-regulated in proportion to the strength of the TCR/self-antigen interaction (5). The elevated expression of CD5 found in V8.3+ CD4+ T cells compared with other murine strains is likely due to the lower intrinsic reactivity of the TS1(SW) TCR for the S1 peptide.
compared the proliferation of purified Vα8.3+ CD4+ T cells from TS1(SW) mice (which are CD5lo) with the proliferation of purified Vα8.3+ CD4+ T cells from TS1(SW)3 HA104 mice (which are CD5hi), in response to S1 or S1(SW) peptides in vitro. The Vα8.3+ CD4+ T cells from TS1(SW) × HA104 mice were less sensitive to both S1 and S1(SW) peptides than those from TS1(SW) mice, requiring ~3-fold higher concentrations than cells from TS1(SW) mice to achieve half-maximal stimulation with either peptide (Fig. 3A). This decreased sensitivity of the CD5hi Vα8.3+ CD4+ T cells from TS1(SW)3 HA104 mice is consistent with studies showing that CD5 can be a negative regulator of TCR signaling (29).

We next examined the ability of Vα8.3+ CD4+ T cells from TS1(SW) versus TS1(SW) × HA104 mice to proliferate in response to S1 peptide presented in vivo in HACII and HA104 mice. When transferred into HACII hosts, CFSE-labeled Vα8.3+ CD4+ T cells from both TS1(SW) and TS1(SW) × HA104 mice underwent division (Fig. 3B). However, the Vα8.3+ CD4+ T cells from TS1(SW) × HA104 mice (which are CD5hi) underwent a modestly reduced degree of division than their counterparts from TS1(SW) mice (which are CD5lo). Strikingly, neither population of Vα8.3+ CD4+ T cells underwent more division in response to S1 peptide following transfer into HA104 mice than they underwent following transfer into BALB/c mice (Fig. 3B). We also performed similar studies in BALB/c and HA104 mice that had been rendered lymphopenic by sublethal irradiation. Under these conditions, the Vα8.3+ CD4+ T cells from both TS1(SW) and TS1(SW) × HA104 mice underwent homeostatic proliferation to equal extents in BALB/c mice, and neither population underwent enhanced proliferation in response to S1 peptide in the lymphopenic environment (Fig. 3C). Thus, Vα8.3+ CD4+ T cells from TS1(SW) × HA104 mice had increased CD5 expression in response to S1 self-peptide, and these CD5hi T cells exhibit reduced proliferative responses to S1 peptide in vitro as well as in vivo in HACII mice. However, this enhanced CD5 expression does not appear to prevent proliferative responses to the S1 self-peptide in HA104 mice because Vα8.3hi CD4+ T cells from TS1(SW) mice (which are CD5lo) appear no more responsive to S1 peptide in HA104 mice than are

Fig. 2. The effect of TCR reactivity on CD4+ T cell selection and phenotype. (A) Bar graphs indicating the total cellularity, CD4SP thymocyte number and CD4SP clonotype+ thymocyte number from TS1, TS1 × HA104 and TS1 × HACII mice, and in TS1(SW), TS1(SW) × HA104 and TS1(SW) × HACII mice. (B) Histograms represent the expression of the 6.5 TCR on CD4SP thymocytes from TS1 (thin line) versus TS1 × HA104 or TS1 × HACII mice lineages (bold line) (left panels) or the Vα8.3 TCR on CD4SP thymocytes from TS1(SW) (thin line) versus TS1(SW) × HA104 or TS1(SW) × HACII mice (bold line) (right panels). Histograms are representative of at least three independent experiments. (C) Histograms indicate the level of CD5 expression Vα8.3+ CD4SP thymocytes or Vα8.3+ CD4+ T cells from TS1(SW) mice (dotted line) versus TS1(SW) × HA104 or TS1(SW) × HACII mice (thin line). The MFI values of CD5 expression in one representative experiment out of four are indicated in each histogram, with values from TS1(SW) × HA104 or TS1(SW) × HACII mice in each case shown in bold.
It was noteworthy that the Vα8.3hi CD4+ T cells from TS1(SW) × HA104 mice, although modestly hyporesponsive, nevertheless proliferated readily in response to their cognate S1(SW) peptide in vitro (Fig. 3A). Previous studies have shown that effector CD4+ T cells can possess increased sensitivities to stimulation through their TCR, relative to naive T cells (13–15). We therefore wanted to determine whether effector Vα8.3+ CD4+ T cells generated from TS1(SW) × HA104 mice are able to proliferate in response to the S1 neo-self-peptide in vitro. Accordingly, effector CD4+ T cells were generated by incubating CD8-depleted LN cells from TS1(SW) × HA104 mice with S1(SW) peptide and APCs. We first compared naive and effector Vα8.3+ CD4+ T cells from TS1(SW) × HA104 mice for their ability to proliferate in response to graded doses of S1 and S1(SW) peptides in vitro. The Vα8.3+ CD4+ effector T cells were approximately 3- to 10-fold more sensitive to both the S1 and S1(SW) peptides than their naive counterparts (Fig. 4A), consistent with previous studies showing that effector T cells exhibit increased sensitivity to TCR signaling (13, 14, 16).

We next examined how this increased sensitivity might affect the ability of Vα8.3+ CD4+ T cells from TS1(SW) × HA104 mice to proliferate in response to S1 peptide presented in vivo. CFSE-labeled Vα8.3+ CD4+ effector T cells from TS1(SW) × HA104 mice underwent much more division than naive T cells in response to S1 peptide in HACII mice, again indicating that these effector T cells were more sensitive to stimulation via the TCR (Fig. 4B). Notably, many of the Vα8.3+ CD4+ effector cells from TS1(SW) × HA104 mice underwent a round of proliferation in HA104 mice, unlike naive Vα8.3+ CD4+ T cells in which the transferred cells showed no more evidence of proliferation than occurred in BALB/c mice (Fig. 4B). Thus, the increased sensitivity of the TS1(SW) TCR when expressed by effector CD4+ T cells, relative to naive CD4+ T cells, caused Vα8.3+ CD4+ effector T cells from TS1(SW) × HA104 mice to undergo division in response to the S1 self-peptide in HA104 mice.

**Fig. 3.** Proliferative responses of Vα8.3+ CD4+ T cells from TS1(SW) and TS1(SW) × HA104 mice. (A) FACS-purified Vα8.3+ CD4+ LN cells from TS1(SW) (diamonds) or TS1(SW) × HA104 (circles) mice were stimulated with graded doses of S1 (filled symbols) or S1(SW) (open symbols) peptide. Proliferation determined by [3H]-thymidine incorporation is shown. Averages ± SD of triplicate wells from a single experiment are shown. Data are representative of three independent experiments. (B) CFSE-labeled LN cells from TS1(SW) or TS1(SW) × HA104 mice were adoptively transferred into BALB/c, HA104 or HACII hosts. Seven days later (BALB/c and HA104 hosts) or 3 days later (HACII hosts), LN cells from each host were harvested and stained with anti-CD4 and anti-Vα8.3 antibodies. Histograms show CFSE levels of Vα8.3+ CD4+ T cells from TS1(SW) (left column) or TS1(SW) × HA104 (right column) mice and are representative of three independent experiments. (C) CFSE-labeled LN cells from TS1(SW) or TS1(SW) × HA104 mice were adoptively transferred into sublethally irradiated (700 rad) BALB/c or HA104 mice. Seven days later, LN cells were stained with anti-CD4 and anti-Vα8.3 antibody for FACS analysis. Histograms show CFSE levels on Vα8.3+ CD4+ LN cells and are representative of four independent experiments.
Effects of TCR reactivity on B cell activation in vivo

We also examined CD4+ T cells from TS1 and TS1(SW) mice for their ability to activate B cells from HACII mice, which express the PR8 HA as a cell-surface antigen on B cells and other MHC class II+ cells such as dendritic cells (17, 24). As described above, both 6.5+ T cells from TS1 mice and Vα8.3+ T cells from TS1(SW) and TS1(SW) × HA104 mice proliferate following transfer into HACII mice (although not equally, with
the higher reactivity 6.5+ CD4+ T cells undergoing more proliferation). Five days after transfer of naive CD4+ T cells from TS1 mice, B cells from recipient HACII mice expressed higher levels of both B7.2 and I-A^d than were found on B cells from BALB/c recipient mice, indicating that interactions between 6.5+ CD4+ T cells and S1 peptide can lead to B cell activation (30). By contrast, no increases in these molecules' expression level were found on B cells from mice receiving naive CD4+ T cells from TS1(SW) or TS1(SW) × HA104 mice (Fig. 5A). However, CD4+ T cells from either TS1(SW) or TS1(SW) × HA104 mice that had been expanded in vitro with S1(SW) peptide under conditions that promoted Th2 differentiation were able to promote up-regulation of both B7.2 and I-A^d with comparable efficiency to naive CD4+ T cells expressing the higher reactivity TS1 TCR (Fig. 5B), while those that had undergone Th1 differentiation did not acquire this capacity.

To examine the abilities of each of the T cell populations to provide help for HA-specific B cell responses, we transferred CD4+ T cell-depleted splenocytes obtained from BALB/c mice that had been primed 1 month earlier with PR8 virus to provide a source of PR8 HA-specific B cells along with the CD4+ T cell populations into HACII mice. Sera from HACII mice that received CD4+ T cells from TS1 mice contained increased levels of HA-specific antibody relative to BALB/c mice (Fig. 6A). The formation of HA-specific antibody depended on the presence of both TS1 CD4+ T cells and HA-specific B cells, and also required the presence of the neo-self-HA to activate the HA-specific CD4+ T and B cells (because antibody formation did not occur in BALB/c mice that received splenocytes and T cells). No increases were found in the levels of HA-specific antibody in mice receiving naive CD4+ T cells from either TS1(SW) or TS1(SW) × HA104 mice (Fig. 6A). However, effector CD4+ T cells that had undergone Th2 (but not Th1) differentiation promoted HA-specific antibody production at comparable levels to those induced by CD4+ T cells expressing the 6.5 TCR (Fig. 6B and C). Collectively, these data indicate that the low intrinsic reactivity of the TS1(SW) TCR for the S1 self-peptide limits its ability to signal naive CD4+ T cells to provide help for B cell responses. However, following differentiation to a Th2 phenotype, cells expressing the TS1(SW) TCR can provide B cell help with comparable efficiency to T cells expressing the TS1 TCR.

**Discussion**

In this study we have examined how the varying reactivities of TCRs can exhibit toward peptides affects the functional potential of autoreactive CD4+ T cells. Overall, the results show that the low reactivity TS1(SW) TCR can evade deletion by the S1 self-peptide as it is expressed in HA104 mice, while the higher reactivity TS1 TCR is substantially deleted in TS1 × HA104 mice. Evasion from deletion by the TS1(SW) TCR is

![Fig. 5. Effects of TCR reactivity on B cell activation in HACII mice.](image)

(A) Expression of B7.2 and MHC class II molecule I-A^d on B220+ splenocytes of BALB/c (dotted line) or HACII mice (black thin line) 5 days after adoptive transfer of naive LN cells from TS1 mice (upper histograms), TS1(SW) (middle histograms) or TS1(SW) × HA104 (bottom histograms) mice. Histograms show levels of B7.2 and I-A^d on B220+ cells. Data are representative of at least three independent experiments. (B) Th2-deviated CD4+ T cell effectors from TS1(SW) or TS1(SW) × HA104 mice were transferred into HACII (bold line) or BALB/c (dotted line) mice. Histograms show expression of B7.2 and I-A^d on B220+ cells. Data are representative of at least three independent experiments. (C) Th1-deviated CD4+ T cell effectors from TS1(SW) or TS1(SW) × HA104 mice were transferred into HACII (bold line) or BALB/c (dotted line) mice. Histograms show expression of B7.2 and I-A^d on B220+ splenocytes 5 days following transfer; expression levels in mice that did not receive transferred cells are also shown (solid line). Data are representative of at least three independent experiments.
associated with increased levels of CD5 and a modest degree of hyporesponsiveness to the self-peptide. Nonetheless, clonotypic CD4+ T cells from TS1(SW) × HA104 mice can be readily activated by a homolog of the S1 self-peptide. Moreover, and in contrast to naive T cells, effector CD4+ T cells from TS1(SW) × HA104 mice acquire the ability to proliferate and provide B cell help in response to the S1 neo-self-peptide in vivo.

The finding that Vα8.3+ CD4+ T cells underwent little or no deletion in TS1(SW) × HA104 mice and expressed increased CD5 levels resembles observations in other systems in which CD5 up-regulation has been associated with a non-deletional interaction with a self-antigen (5, 26–28). Moreover, ongoing interactions in the periphery with the selecting antigen appear to be required to maintain CD5 up-regulation on mature CD4+ T cells, as was observed in Vα8.3+ CD4+ T cells from TS1(SW) × HA104 mice (31). Since CD5 is a negative regulator of TCR signaling, these previous observations have been interpreted as indicating that CD5 can play a role in ‘fine-tuning’ the responsiveness of T cells that may possess an intrinsic reactivity toward a self-antigen that falls below a threshold necessary for negative selection, and CD5 up-regulation may prevent responses to these self-antigens by peripheral CD4+ T cells that have evaded deletion (26–28). We found that the CD5hi Vα8.3+ CD4+ T cells from TS1(SW) × HA104 mice were ~3-fold less sensitive to stimulation with either S1(SW) or S1 peptides in vitro than their CD5lo counterparts from TS1(SW) mice. In addition, the CD5hi Vα8.3+ CD4+ T cells from TS1(SW) × HA104 mice appeared to be less proliferative following transfer into HACII mice, in which S1 peptide expression is targeted to APCs. Thus, the up-regulation of CD5 on Vα8.3+ CD4+ T cells from TS1(SW) × HA104 mice was associated with a decreasing responsiveness to S1 peptide that in magnitude to that described in other studies analyzing effects of CD5 up-regulation (27, 31). Significantly, in the studies here, we were able to directly examine whether reduced TCR sensitivity accompanying CD5 up-regulation was important in preventing proliferative responses to the S1 peptide in HA104 mice, i.e., in the environment in which interactions with S1 peptide had induced CD5 up-regulation.

Previous studies have shown that effector CD4+ T cells are more sensitive to activation via the TCR than are naive CD4+ T cells, and we examined whether effector CD4+ T cells from TS1(SW) × HA104 mice exhibited increased proliferation in HA104 mice relative to BALB/c mice, which lack the S1 peptide. Thus, our findings suggest that CD5 up-regulation is not necessary to prevent CD4+ T cells expressing the TS1(SW) TCR from proliferating in response to S1 peptide in TS1(SW) × HA104 mice, even though cells expressing this TCR up-regulated CD5 in response to S1 peptide during their development. The findings further suggest that the threshold of reactivity that is necessary to induce CD5 up-regulation is substantially lower than that required to induce proliferation of CD4+ T cells, because even CD5lo Vα8.3+ CD4+ T cells from TS1(SW) × HA104 mice failed to proliferate in response to the S1 peptide in HA104 mice. Moreover, and in contrast to naive T cells, effector CD4+ T cells from TS1(SW) × HA104 mice acquire the ability to proliferate and provide B cell help in response to the S1 neo-self-peptide in vivo.
response to S1 peptide in HACII mice, where it is expressed by APCs. Unlike their naive counterparts, effector Vα6.3+ CD4+ T cells from TS1(SW) × HA104 mice underwent proliferation when transferred into HA104 mice, but not following transfer into BALB/c mice. Thus, the increased sensitivity of effector T cells to activation allowed CD4+ T cells that evaded negative selection by S1 peptide due to their low intrinsic reactivity for this peptide to become able to respond to the S1 peptide by proliferation. Moreover, when induced to undergo differentiation to become T$_{h2}$ effectors, the CD4+ T cells from TS1(SW) × HA104 mice became efficient inducers of B cell activation and antibody secretion under circumstances that depended on their ability to react with S1 peptide. Previous studies have shown that effector CD4+ T cells can provide faster, more effective responses to peptide stimulation and are less dependent on co-stimulation than naive T cells (14–16). In addition, effector CD4+ T cells can secrete cytokines and express other molecules that increase their ability to provide help for different kinds of immune response (e.g. T$_{h2}$ cells can promote humoral immunity) (32). The studies here provide evidence that these qualitative changes can also increase the capacity of CD4+ T cells with low reactivity for self-peptides to provide effective help for B cell responses, when differentiated to become T$_{h2}$ effector T cells. In this regard, it is noteworthy that the T$_{h1}$ effector cells expressing the low reactivity TS1(SW) TCR did not provide efficient help for B cell responses in this system. It may be that differences in co-stimulatory molecules, cytokine secretion, cell trafficking or survival limit the ability of T$_{h1}$ versus T$_{h2}$ cells to provide help for B cells in this system. Indeed, our findings suggest that for CD4+ T cells that have low intrinsic reactivities for self-peptides, the acquisition of specialized T$_{h2}$ effector function is crucial in their ability to provide help for B cell responses.

Collectively, these studies suggest that the varying sensitivity of CD4+ T cells to stimulation via the TCR at distinct developmental stages could contribute to the ability of pathogens to activate autoreactive CD4+ T cells in autoimmune disease (molecular mimicry) (12). CD4+ T cells expressing the TS1(SW) TCR possess a level of reactivity with the S1 peptide that is below the threshold necessary to induce overt deletion in TS1(SW) × HA104 mice, but following activation with a viral homolog of the S1 peptide against which they are more highly reactive, they acquired reactivity toward S1. In addition, it is notable that the T$_{h2}$-deviated CD4+ T cells from TS1(SW) × HA104 mice appeared to provide help as efficiently as naive CD4+ T cells from TS1 mice, even though they exhibited a much more modest proliferative response to S1 peptide in vivo. Based on these studies, then, the acquisition of a differentiated phenotype (e.g. ability to provide B cell help) may be more important than enhanced proliferation in allowing CD4+ T cells that evade negative selection to participate in autoimmune responses.

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>American Type Tissue Collection</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HAU</td>
<td>hemagglutinating units</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>PR8</td>
<td>influenza virus A/PR/8/34</td>
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<tr>
<td>SW</td>
<td>influenza virus A/SW/33</td>
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References

with a class II major histocompatibility complex-restricted receptor. 


